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Semiconductor laser-induced fluorescence detection in capillary electrophoresis using a cyanine dye

Fu-Tai A. Chen*, Anton Tusak, Stephen Pentoney, Jr., Ken Konrad, Clarence Lew, Ed Koh and James Sternberg

Beckman Instruments Inc., 2500 Harbor Boulevard, Fullerton, CA 92634 (USA)

ABSTRACT

Cy5, an activated carboxyl cyanine fluorophore, was characterized by capillary electrophoresis (CE) using a semiconductor laser at 652 nm to induce fluorescence. Hydrolysis of the activated Cy5 in the presence of ammonia results in the formation of a mono- and diamide and a dicarboxylic acid. A Cy5-labeled oligonucleotide M_{13} primer for DNA sequencing (M13mp18 template) was synthesized with a purity of better than 95%. The labeled primer was analyzed by liquid chromatography, using UV–visible detection, and by CE, monitored by laser-induced fluorescence (LIF) detection. Analysis of the Cy5-labeled oligonucleotide primer by CE–LIF in a 9% polyacrylamide gel-filled capillary indicated the purity of the major Cy5–oligonucleotide primer was greater than 90%. The detection sensitivity for Cy5-based CE–LIF detection system with a 2.5-mW red semiconductor laser is about 10^{-10} *M*.

INTRODUCTION

Cy5 (Biological Detection Systems, Pittsburgh, PA, USA) is an activated cyanine dye that can be readily coupled with oligonucleotides, peptides and proteins [1,2]. It contains two broad absorption maxima, at 630 and 655 nm ($\epsilon = 150\,000$ and $215\,000$ cm^{-1} M^{-1}, respectively). The high extinction coefficient and fluorescence quantum yield of the Cy5, with its absorption maximum at 655 nm, appeared to make the dye suitable for laser-induced fluorescence (LIF) detection using a semiconductor laser source emitting at 652 nm. The following report deals with the characterization of Cy5 and its derivatives by means of the capillary electrophoresis (CE) technique using a red semiconductor laser and LIF detection. The synthesis of the Cy5-labeled oligonucleotide, a 20-mer M_{13} primer, will also be described. The Cy5-labeled oligonucleotide may potentially be used as a DNA hybridization probe and for sequencing.

* Corresponding author.

EXPERIMENTAL

Capillary electrophoresis procedures

A P/ACE 2100 equipped with a LIF detection system by Beckman Instruments (Fullerton, CA, USA) was used with P/ACE system software controlled by an IBM PS/2 Model 55 SX. Post-run data analysis was performed on System Gold software by Beckman Instruments. An open capillary column, typically of 27 cm length (20 cm to detector window) × 75 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA) was assembled in the P/ACE cartridge format (100 × 200 μm aperture). The buffer used in the open capillary column was 80 m*M* borate, pH 10.0 [3]. The gel-filled capillary was prepared according to the procedure of Heiger *et al.* [4], and modified by Pentoney *et al.* [5]. A 2.5-mW diode laser

emitting at 652 nm and a 10-mW red helium–neon laser emitting at 632.8 nm were purchased from Melles Griot, Irvine, CA, USA. Both the laser headcoupler and the standard SMA-905 fiber connectors were purchased from the OZ optics, Ontario, Canada.

Cy5-labeled oligonucleotide primer

Cy5 was a product of Biological Detection Systems (BDS). M13 primer, ACGTTGT-AAAACGACGGCCA, (80 nmole of 20-mer) with a hexylamino terminus at its 5′-end, was obtained from Genosys, Houston, TX. The 5′-amino-M13 primer was dissolved in 200 μl of water. A 50-μl (20 nmole) aliquot was added to an acetonitrile solution containing Cy5 (80 nmol/100 μl acetonitrile) and allowed to stand at room temperature for 60 min. The resulting products were purified on a C_{18} reversed-phase column (25 cm × 4.1 mm) using a combination of methanol and 20 m*M* phosphate buffer at pH 6.0, on a Beckman System Gold LC with a Model 168 diode array detector. Peaks containing both nucleotide bases (260 nm) and Cy5 (655 nm) at a ratio of approximately 1 to 1.4 were collected. Purity was assessed by LC, CE and UV–visible spectra, as shown in the text.

RESULTS AND CONCLUSIONS

Cy5 is an activated dicarboxyl derivative of a cyanine compound that fluoresces in the near infrared region; its structure is shown in Fig. 1. The fluorescence emission maximum of Cy5 in aqueous solution is at 670 nm with a quantum efficiency of 0.13 [1,2]. Hydrolysis of Cy5 in the presence of 0.1 *M* hydrogencarbonate buffer at pH 9.0 yields Cy5–dicarboxylic acid. Using a red helium–neon laser (632.8 nm), the electropherogram of the Cy5–dicarboxylic acid, shown in Fig. 2A, appears to be fairly homogeneous. The addition of concentrated ammonia to the activated Cy5 results in the formation of Cy5-mono- and diamide along with Cy5–dicarboxylic acid (Fig. 2B), consistent with the structure suggested by Southwick *et al.* [1].

A red semiconductor laser that emits at 652 nm was coupled with the CE system for the LIF detection of the Cy5 and its derivatives. The emission spectrum of the red semiconductor

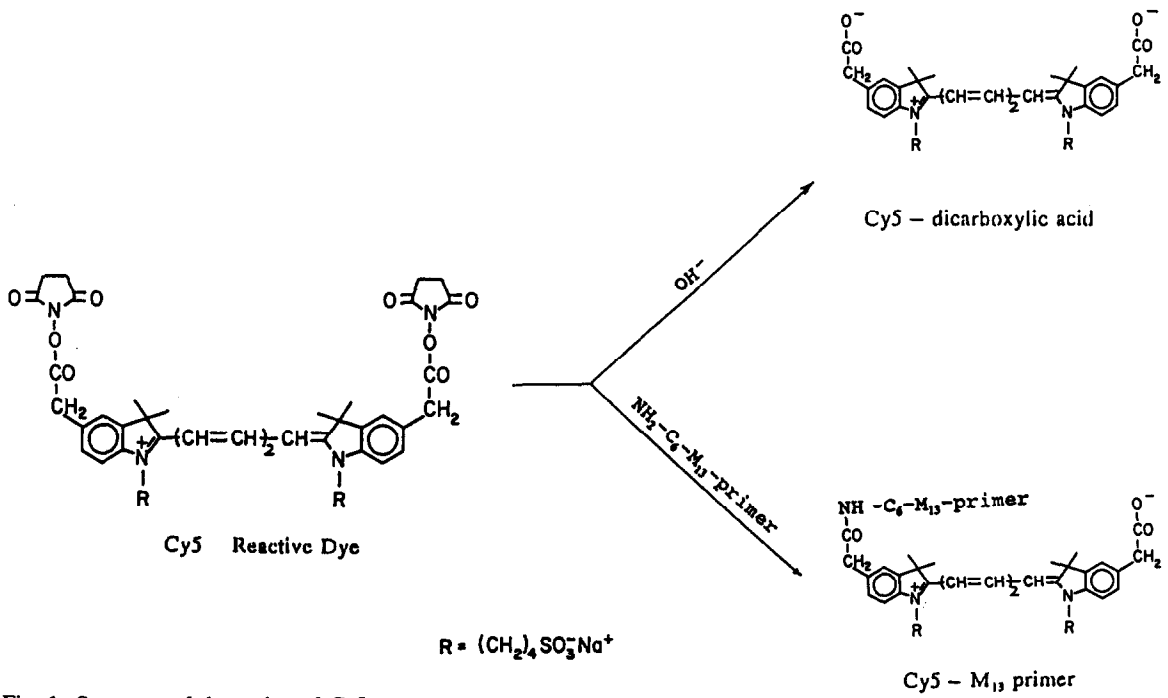

Fig. 1. Structure of the activated Cy5.

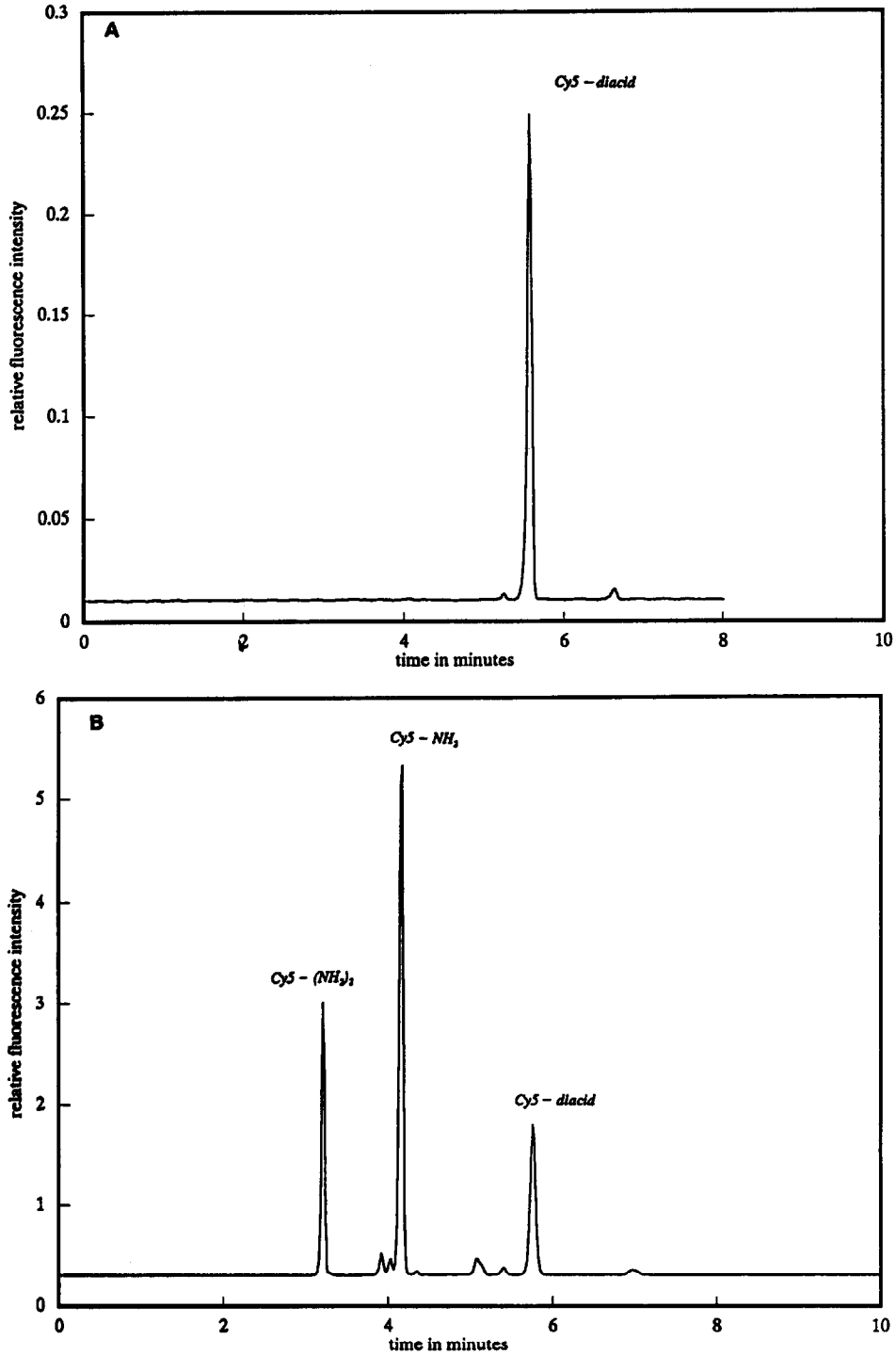

Fig. 2. (A) Electropherogram of hydrolyzed Cy5 at 1.0 n*M*. Conditions: Untreated fused-silica capillary, 25 cm × 75 μm I.D.; light source: 10-mW red helium–neon laser; emission filter: 670 nm ± 10 nm (Oriel, Stratford, CT, USA) and a notch filter at 633 nm (Barr Associates, Westford, MA, USA); applied potential/current: 7 kV/75 μA; buffer: 80 m*M* borate at pH 10.0. (B) Electropherogram of ammonia-treated Cy5 at 20 n*M*. Conditions as in (A).

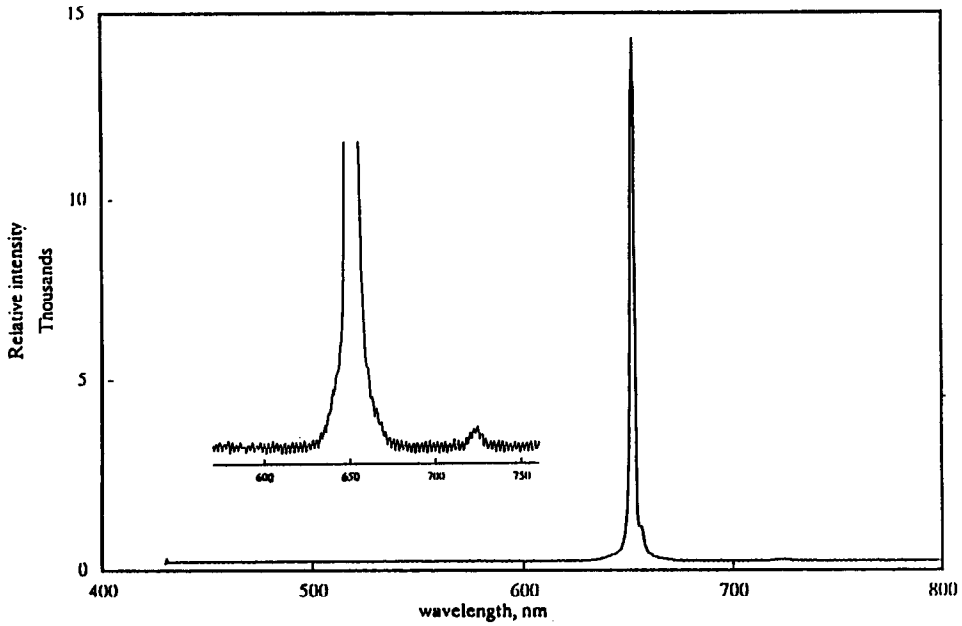

Fig. 3. Spectrum of the red semiconductor laser.

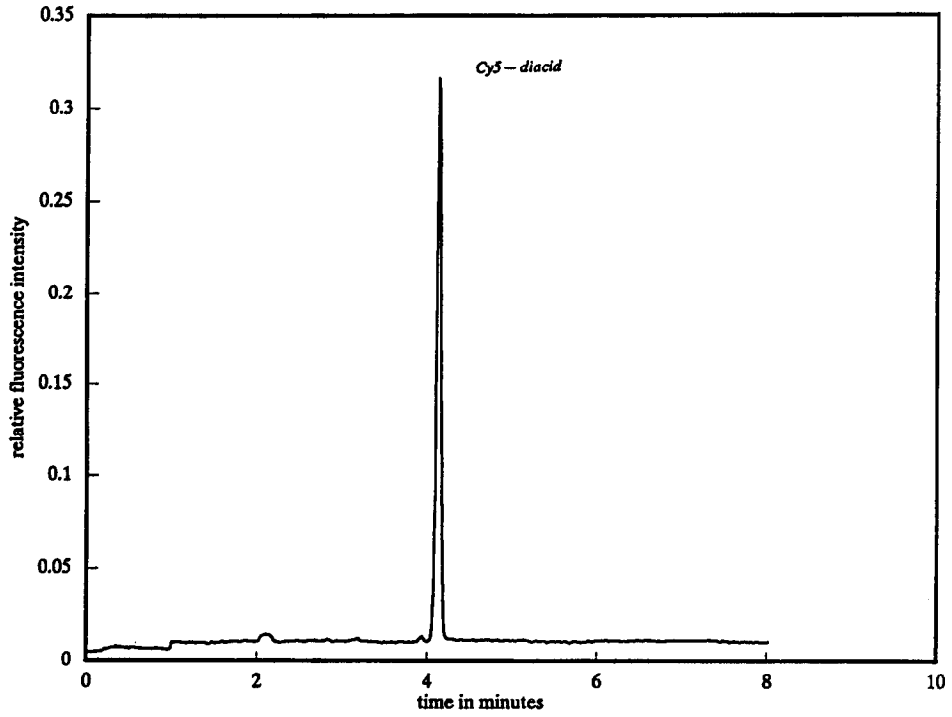

Fig. 4. Electropherogram of hydrolyzed Cy5 at 10 n*M*. Conditions: Untreated fused-silica capillary, 21 cm × 75 μm I.D.; light source: 2.5-mW red diode laser; emission filters: one narrow band filter at 670 nm ± 10 nm (No. 53965) and one long pass filter (No. 51340) (Oriel, Stratford, CT, USA); applied potential/current: 7 kV/82 μA; buffer: 80 m*M* borate at pH 10.0.

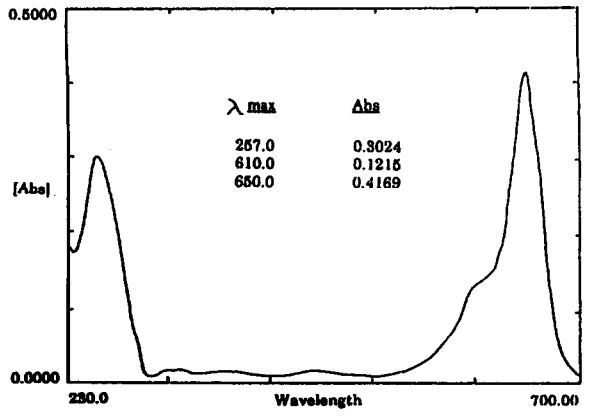

Fig. 5. UV–Vis spectrum of Cy5–M_{13} primer.

laser shown in Fig. 3 was analyzed at room temperature (22–24°C). The emission maximum occurs at 652 nm. A minor emission band at 722 nm was evident upon increasing the detection sensitivity, as shown in the insert in Fig. 3. To detect a fluorescence signal from Cy5 and its derivatives induced by the red diode laser, we have attempted to use the combination of a narrow band filter at 670 ± 10 nm and a long pass filter (cut-off at 660 nm). The electropherogram of a 10 n*M* solution of Cy5–dicarboxylic is shown in Fig. 4.

The synthesis of the Cy5-labeled 5′-amino-oligonucleotide primer was achieved by mixing a four-fold excess molar ratio of the activated Cy5 with 5′-aminohexyl-derivatized oligonucleotide primer, as shown in Fig. 1. The resulting mixture was purified on a reversed-phase column. The fraction showing absorbances at both 260 and 650 nm was collected. The total isolated yield from LC is approximately 70%, based on using 20 nmol of starting material, and obtaining 14 nmol of the final labeled primer.

The UV–Vis spectrum of Cy5-labeled primer is shown in Fig. 5 and the ratio of A_{257nm} *vs.* A_{650nm} is 0.725. Using an average molar extinction coefficient of 9000 for each nucleotide, the A_{257nm} of the 20-mer would be 180 000. The calculated ratio of A_{257nm} *vs.* A_{650nm} for the Cy5-labeled primer is 0.84. Thus, the UV–Vis spectrum observed for the Cy5-labeled primer is consistent with the expected values. Furthermore, analysis of the isolated Cy5-labeled primer

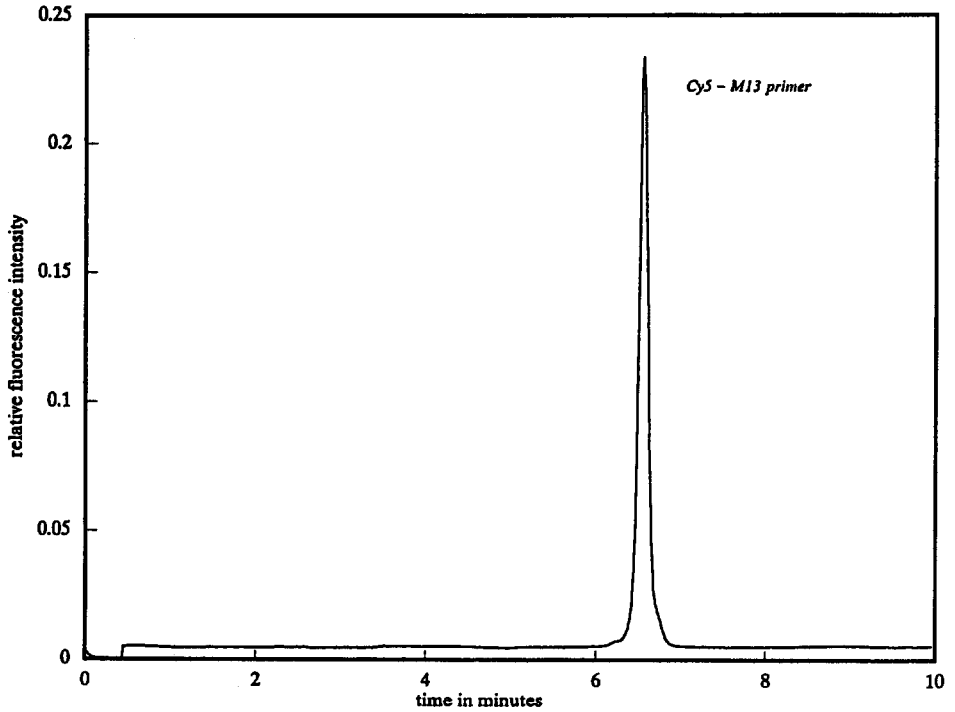

Fig. 6. Electropherogram of Cy5–M_{13} primer at 10 n*M*. Conditions as in Fig. 4.

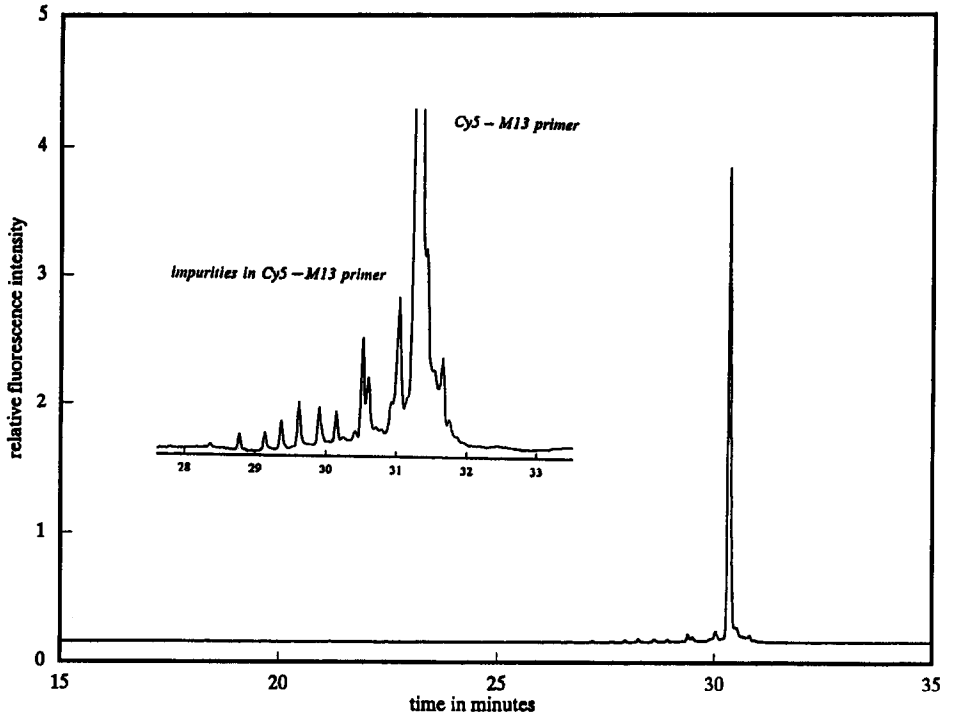

Fig. 7. Electropherogram of Cy5–M_{13} primer at 10 n*M*. Conditions: polyacrylamide gel-filled capillary, 47 cm × 100 μm I.D.; light source: 2.5-mW red diode laser; emission filters: same as those in Fig. 3; applied potential/current: 15 kV/14 μA; buffer: 100 m*M* Tris–borate buffer with 7 *M* urea, pH 8.3.

by CE–LIF in an open tube indicates its purity to be greater than 95% (Fig. 6).

The electropherogram of the labeled primer, obtained using a gel-filled capillary, is shown in Fig. 7. Clearly the isolated labeled primer is about 90% pure, and many small impurity peaks appear to be related to the original amino primer. The impurity peaks are not likely to be associated with the impurities of Cy5 or its hydrolyzed products, since their migration is substantially different from that of the labeled primers and did not exhibit a pattern similar to that shown in Fig. 7.

The detection limit of LIF detection using the red semiconductor laser for Cy5 and its derivatives is about 10^{-10} *M*. A further improvement in the optics may yield a sensitivity of 10^{-11} *M* with Cy5 as the fluorophore.

The functional utility of the Cy5-labeled primer was evaluated in DNA sequencing reactions. Preliminary results indicate that this labeled primer can be used for DNA sequencing by capillary gel electrophoresis using LIF with a red semiconductor laser.[a] Use of the semiconductor laser with Cy5 and its derivatives simplified the instrumentation design, providing a less expensive and more durable light source.

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[a] The results obtained using a 652-nm semiconductor laser for DNA sequencing using a Cy5-labeled primer will be published elsewhere.